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The purpose of this research was to conduct a clinical trial of gene therapy for metastatic breast cancer patients. Surgically removed tumor cells would be maintained in short term culture and then transduced in the laboratory with an adenoviral vector containing the CD80 gene that encodes for the co-stimulatory CD80 molecule (or B7-1 molecule). This molecule is missing from tumor cells but is necessary to initiate an immune response in conjunction with an antigen. The patients then would receive a vaccination into the thigh of their own gene-modified tumor cells that had been irradiated. Demonstrating safety of the vaccine and an immune response to the breast cancer cells were the primary goals of this project. The major findings to date are that breast cancer cells can be successfully maintained in culture. The adenoviral vector transduces all the breast cancer cells and results in strong, long lived expression of the CD80 molecule on the cell's surface. The clinical trial testing the safety and the immune effects of this gene therapy did not begin during the years of this report because the subcontractor did not supply the vector. However, it is now available and the clinical study will begin and be completed.

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In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

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"Gene Therapy of Human Breast Cancer"

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INTRODUCTION

A. PURPOSE AND SCOPE OF THE RESEARCH

The purpose of this research is to develop and clinically test an autologous breast cancer vaccine whose immunogenicity we hypothesize will be significantly enhanced by genetic modification with an adenoviral expression vector for the T cell co-stimulatory molecule, B7-1 (CD80).

The technical objectives of this research as outlined in the original proposal are to:

1. Develop methods to isolate highly purified populations of breast cancer cells from tissue specimens of locally recurrent or metastatic sites obtained from patients with advanced, refractory breast cancer.

2. Study and optimize the efficiency and longevity of B7 expression in breast cancer cells transduced with adenoviral vectors containing the human B7 cDNA.

3. Conduct a Phase I clinical trial assessing the toxicity of autologous B7-transduced irradiated breast cancer cells used as a vaccine to enhance the immune response to the tumor.

4. Perform in vitro and in vivo immunologic monitoring studies on enrolled patients to assess the development of an anti-tumor immune response.

B. SUBJECT OF THIS RESEARCH AND BACKGROUND OF PREVIOUS WORK

1. INVOLVEMENT OF T CELLS IN THE TUMOR RESPONSE.

The importance of T cells in the antitumor immunity has long been appreciated (1). In animal models, T cells have been shown to be critical for the rejection of tumors induced by viruses, chemical mutagens, or ultraviolet irradiation (2-4). Consistent with this, athymic nude mice, which are essentially devoid of functioning T cells, are unable to reject even allogeneic tumors (5). Regarding human tumors, due to the limitations of experimentation, such direct evidence is harder to obtain. Nonetheless, T cell immunosuppression in humans is associated with an increased risk of cancer e.g. patients with AIDS are at risk for the development of Kaposi's sarcoma and non-Hodgkin's lymphoma, and patients receiving immunosuppressive drugs after organ transplantation have an increased incidence of lymphoma and skin cancer (5).

Because T cells are an antigen-specific population of cells, their ability to reject tumors implies that the malignant cells express unique antigens not found on other cells, or that the malignant cells over express antigens that are found on other than normal cells. Although it was commonly held in the past that human cancers did not have tumor specific antigens, recent work has clearly demonstrated their existence. Indeed, a considerable body of evidence indicates that many tumors, including human breast

cancers, express such tumor specific antigens (1, 6-10). For example, patients with breast cancer have been demonstrated to make antigen specific cytotoxic T lymphocytes (CTLs) that recognize certain epitopes of cell-surface mucin proteins that are phenotypically altered through aberrant glycosylation in more than 90% of breast cancers (11-13). The existence of tumor infiltrating lymphocytes which can lyse tumor cells provide another example of the ability of T cells to recognize tumor-associated antigens. Antigen specific T cell responses have also been reported against the non-mutated, self-protein HER-2/*neu* that is overexpressed in 30% of breast cancers (14). Finally, an extreme instance of tumor-specific antigens occurs in the case of allogeneic tumors, which are almost uniformly rejected by immunocompetent hosts. This vigorous immune response may be due to the fact that alloantigens are intrinsically extremely immunogenic (i.e. a 10-100 fold larger percentage of T cells are capable of responding to alloantigens than to nominal antigens) (14).

If human cancers express unique antigens, why aren't they rejected by the immune system? Recent advances in our understanding of the process of T cell activation may provide an answer to this question. It is now appreciated that complete activation of T cells requires 2 signals (15, 16 and below). The first is provided by antigen itself. The second or "co-stimulatory" signal is not antigen specific but is normally provided by bone-marrow-derived antigen presenting cells (APCs) and is required for T cell responses. Moreover, while costimulation of T cells results in their complete activation, presentation of antigen in the absence of this costimulatory signal creates an anergic state whereby the T cells fail to respond normally to the antigen (17, 18). Solid epithelial tumor cells (including breast cancer cells) lack the ability to deliver the costimulatory signal, and we postulate that this induces a state of unresponsiveness in the host that permits the tumor to grow unimpeded by the immune system. Indeed, it can be envisioned that this feature of the immune system, perhaps normally used to inhibit the development of autoimmune disease by preventing a response to self-antigens displayed on non-APCs, might be exploited by malignant cells of epithelial origin. Now that this requirement for a costimulatory signal is known, strategies to provide it can be developed in an attempt to create a vigorous rejection of the tumor by the immune system. The approach presented in this proposal is to use genetic transduction techniques to convert the tumor cell from delivering a tolerogenic signal to an activating signal.

2. THE B7:CD28 INTERACTION CAN PROVIDE A SECOND SIGNAL.

As noted above, activation of T cells through the T cell antigen receptor (TCR) provides the first signal for T cell activation. This is sufficient to lead to the entry of resting G0 cells into the G1 phase of the cell cycle and result in expression of the cell surface high affinity IL-2 receptor (IL-2R). However, in the absence of additional signals, the IL-2 gene is not transcribed, T cells fail to proliferate, and appear to become anergic (reviewed in (15)). Thus, provision of antigen to T cells in the absence of co-stimulation is not a "neutral" event, but can lead to antigen-specific non-responsiveness.

APCs such as macrophages, dendritic cells and activated B cells are capable of providing second signals to T cells, synergizing with TCR stimulation and leading to complete T cell activation. The bulk of accumulated evidence indicates that a membrane bound

molecule present on APCs is capable of providing costimulation to resting T cells or to Th1-type T cell clones (19, 20), and that a second signal is transduced when a T cell accessory molecule binds to its ligand on an APC. Recently, it has been shown that the interaction between CD28 on T cells, and its ligand B7-1 (or CD80) on APCs is capable of delivering a co-mitogenic second signal (21-23).

a. CD28

CD28 is a 44 kd homodimeric glycoprotein member of the immunoglobulin gene superfamily. It is expressed on the surface of 80% of all peripheral T cells (95% of $CD4^+$ cells and 50% of $CD8^+$ cells) (24), and is the surface signal transducing element of a unique T cell activation pathway. Stimulation of T cells via the surface molecule CD28 can provide a second signal capable of synergizing with TCR stimulation to induce mitogenesis (24). Stimulation of the CD28 molecule strongly induces IL-2 production in TCR-stimulated T cells (24).

In addition to this co-mitogenic effect, CD28 stimulation enhances lymphokine gene expression 5-50 fold, even in T cells already maximally stimulated (25). This effect is mediated by specific stabilization of lymphokine mRNA (26). Signal transduction via CD28 also synergizes with the phorbol ester PMA to activate T cells independent of a rise in intracellular calcium (24). Perhaps due to its lack of dependence on calcium flux, T cell activation via CD28 is relatively resistant to the immunosuppressive effects of cyclosporine (24). Recently, studies have shown that stimulation of the CD28 pathway in resting T cells and T cell blasts activates a protein tyrosine kinase which phosphorylates a distinct pattern of substrates than those which are induced following TCR/CD3 stimulation (27).

Finally, as noted above, previous studies have demonstrated that TCR engagement of T cells in the absence of accessory cells, or with accessory cells incapable of providing a second signal, can induce anergy (15). Upon identification of the CD28 pathway, it was postulated that co-stimulation via CD28 might block the development of anergy in this type of system. Harding et al. (28) have recently demonstrated this phenomenon showing that costimulation with anti-CD28 mAb prevents the induction of anergy in murine T cell clones stimulated with antigen in the absence of a second "costimulatory" signal.

b. B7 (CD80)

Like CD28, the B7 molecule is a member of the immunoglobulin gene superfamily. It was first defined as a B cell antigen expressed on activated and neoplastic B cells (29-31). However, B7 expression is not restricted to B-lineage cells, as B7 is inducible on IFNγ or LPS-treated monocytes (32). Thus the pattern of B7 expression parallels the ability of APCs to provide accessory function. Subsequently, B7 was shown to be a natural ligand for CD28 (21). Cloning of the B7 cDNA (35) and its expression in transfected CHO cells (21, 22) has enabled studies of the results of CD28 stimulation by its natural ligand. These have shown that B7 binding to CD28 is capable of replicating many of the previously observed actions of anti-CD28 mAb such as co-mitogenesis with anti-CD3 mAbs or PHA, stimulation of IL-2 gene expression, and induction of protein tyrosine phosphorylation (21, 22, 27). Further

study has also indicated that the cognate interaction between CD28 on $CD8^+$ cytotoxic T lymphocytes (CTLs) and B7 on target cells is an important component of the in vitro cytolytic response, and blockade of this interaction can significantly inhibit target cell lysis (33). These observations are important in light of recent data regarding the use of B7 to induce tumor specific immunity (see below).

c. Other ligands for CD28.

Recently, several groups have reported that B7-1 is not the sole ligand for CD28, but rather that there is at least one related molecule, termed B7-2 (or CD86), which can also function as a stimulatory ligand for CD28 (34-37). The expression pattern of B7-2 differs somewhat from B7-1 in that it is expressed at higher levels on antigen presenting cells, and is induced at earlier time points in activated cells. From a functional standpoint, there is no known difference between activation of the CD28 pathway via ligation with B7-1 versus ligation with B7-2. Both CD28 ligands provide costimulatory signals to T cells. While the distinct physiologic roles of B7-1 and B7-2 remain to be elucidated, it is clear that both molecules can provide second signals to T cells. While the choice to use B7-1 for adoptive immunotherapy was based on its identification at the time as the only ligand for CD28, the data regarding the use of B7-1 to induce tumor rejection (Townsend, Chen, Baskar, and the preliminary data shown below) validates this choice.

3. THERAPEUTIC APPROACHES TO INDUCE TUMOR IMMUNITY.

a. Systemic IL-2 +/- LAK cells

The immune response to foreign antigens involves a coordinated release of cytokines from T cells which induces the clonal expansion and/or activation of a variety of effector cells including CTLs, B cells, NK cells, and macrophages. A variety of strategies have been devised to induce tumor-specific immunity based on the notion that defective production of cytokines may play a role in the failure of animals to "reject" their tumor. Thus, exogenous systemic administration of lymphokines such as IL-2 with or without adoptively transferred lymphokine activated killer cells (LAK cells) can be an effective form of anti-tumor therapy for patients with renal cell carcinoma, melanoma or lymphoma (38). Although the exact mechanism of action of IL-2 is not known, it is well established that IL-2 has no direct antiproliferative activity, so its ability to induce tumor regressions in patients with advanced disease indicates it is working indirectly, perhaps by enhancing the immunogenicity of the cancer, the cytotoxicity of immune effector cells or through the induction of other directly antiproliferative cytokines. Unfortunately, systemic administration of high doses of IL-2 is associated with severe side effects and many prevalent cancers such as colon, lung and breast cancer fail to respond significantly to this treatment.

b. Systemic IL-2 + Tumor Infiltrating Lymphocytes (TILs).

Animal experiments demonstrated that TILs, which are antigen specific cytotoxic lymphocytes, are 100 times more potent than the non-MHC restricted LAK cells in adoptive immunotherapy studies (39). Culturing TILs with IL-2 resulted in their expansion and enhancement of their ability to kill in an antigen restricted fashion (39). In mice, these in vitro stimulated TILs mediated tumor regression when

adoptively transferred into syngeneic hosts inoculated with the specific tumor cell line from which the TILs were originally derived (39). Preliminary results using a similar approach in patients with metastatic melanoma have yielded promising results (40). Responses appear to correlate with the number of TILs administered and to the specific cytotoxicity in vitro of the TILs to the autologous tumor (41, 42). However, therapy utilizing the adoptive transfer of TILs is technically cumbersome, expensive, and fails to produce TILs in approximately one third of the patients. The vaccination approach in this research project should also lead to the development of TILs in the draining lymph nodes. Future therapeutic protocols could attempt to harvest and expand those TILs for adoptive immunotherapy.

c. Vaccination with genetically altered cells

Gene transfer into tumor tissue has become a potentially attractive means of enhancing the immune response to the tumor. To date, a variety of cytokine genes including IL-2, TNF, IFNγ, GM-CSF, and IL-4 (43-49) have been transfected into tumor cells ex vivo in an attempt to overcome the lack of effective T cell stimulation by creating a local continuous secretion of a cytokine that will activate T cells, recruit other inflammatory cells into the area and possibly induce other cytokines to be secreted. Animal studies have shown that this approach enhances the immunogenicity of the tumor, limiting its outgrowth and in some cases, causing established tumors to regress. Importantly, in some instances (IL-2, TNF, and IL-4), mixing both non-transfected and transfected cells prior to injection at a single site resulted in regression of both cell populations, indicating that the induced immunity was locally active. Another approach is to directly transduce a gene into tumor cells that encodes a foreign MHC molecule to enhance the recognition of the tumor cell with the hope of increased local cytokine production and T cell activation (50). The early toxicity trials of these gene therapy approaches are currently underway.

The strategy pursued in this research will be to genetically alter breast cancer cells to express a gene which encodes for the cell surface protein, B7-1 (CD80) which provides a potent costimulatory signal for $CD4^+$ cells to produce a variety of lymphokines, and which also appears to be capable of directly stimulating $CD8^+$ CTLs to lyse targets. The advantage this approach might have over the expression of a single cytokine gene is it should recreate the normal scenario by which the immune system detects and destroys cells bearing foreign antigens. That process involves a complicated orchestration of the production of multiple cytokines and cell-to-cell interactions. We feel that transducing a gene to provide a costimulatory signal to T cells will more likely result in the coordinated secretion of multiple cytokines in the proportions that are efficacious in generating immunity and therefore, may be more therapeutically effective than the local production of a single cytokine gene. This precise strategy (transduction of B7) already has been used in animal experiments by other groups to induce tumor specific immunity to melanoma or to sarcoma (51-53). Our own preliminary studies indicate that this approach is also successful in inducing rejection of murine breast cancer cell lines (see below).

4. USE OF B7 TO INDUCE TUMOR REJECTION.

Three different groups of investigators have conducted animal experiments that demonstrate an important role for B7 in inducing antitumor immunity in vivo. Townsend and Allison (51) transfected a K1735 murine melanoma cell line with murine B7 and injected it into syngeneic mice. Compared to a control vector-transfected K1735, the B7-transduced tumor grew less well as mice injected with the B7-expressing tumor were able to "reject" the malignant cells and appeared disease free. Furthermore, prior exposure to the B7-transfected tumor cells was able to protect the host mice from a subsequent challenge of the parental tumor cell line. The effect appeared to be dependent on the presence of $CD8^+$ T lymphocytes, because B7-expressing cells grew readily in mice depleted of this subset, with a tumor growth rate the same as that observed with the parental cell line or the vector-transduced control tumor cell line. Depletion of $CD4^+$ T cells did not affect the ability of the host animals to reject their tumor.

Chen et al. (52) used the K1735 cell line that was further modified by insertion of a xenogeneic viral antigen, the E7 gene product of human papilloma virus. They transduced this modified cell line with B7 and showed that immunization with the B7-transduced cell line protected animals from subsequent challenge of the parental tumor cell line and also from challenge with non-B7-transduced tumor cells, indicating that it may not be necessary to transduce all of the tumor cells with B7 to elicit an immune response against the established tumor. Again, the antitumor activity seemed to be mediated by $CD8^+$ T lymphocytes. These investigators also showed that injection of B7-transduced tumor cells in one flank resulted in the complete rejection of non B7-transduced tumor cells placed simultaneously in the other flank. Finally, this treatment was shown to eliminate established pulmonary micrometastases from non B7-transfected tumor cells.

In the third study (53), Glimcher and colleagues demonstrated that mouse sarcoma cells genetically engineered to express B7 stimulated potent tumor-specific T cells that caused rejection of both transfected and native neoplastic cells. A notable difference between this study and the other two was that $CD4^+$ T cells were responsible for the antitumor activity in this model. It is also noteworthy that this study utilized sarcoma cells rather than melanoma cells, thus showing that the use of B7 to induce tumor rejection can be extended to tumors other than melanoma.

In summary, these studies make several important points. First, B7 expression by itself can lead to tumor rejection. Second, rejection of a B7-positive tumor can lead to protective immunity against a B7-negative tumor. Third and most important for clinical use, exposure to a B7-expressing tumor can lead to rejection of previously established metastatic B7-negative tumor.

5. USE OF B7 TO INDUCE PROTECTIVE IMMUNITY AGAINST BREAST CANCER.

Our collaborators James Wilson, Steven Eck, and William Lee at the University of Pennsylvania have performed a series of preliminary studies designed to examine the use of ectopic B7 expression in a murine model of breast cancer. SCK cells (J.G. Rhee, University of Maryland, Baltimore, MD) arising from a spontaneously mammary carcinoma in A/J mice ($H\text{-}2^a$) is a murine mammary carcinoma cell line that grows in

culture as well as forms lethal tumors in syngeneic hosts. After subcutaneous inoculation of as few as 1.0 x 10^3 viable SCK cells into 6-8 week-old, female, syngeneic mice tumors are consistently detectable within three weeks. Death or the need for euthanasia inevitably follows about 7 to 10 days later.

SCK cells normally do not express cell surface B7 antigen. To test the effect of ectopic B7 expression on in vivo tumor cell growth, SCK cells that constitutively express murine B7 (mB7) were generated. A cDNA encoding normal mB7 (from Louis Lanier, DNAX, Palo Alto, CA) was inserted into the pMV6 proviral vector. pMV6 and pMV6mB7 were transduced into ψCre packaging cells to generate ecotropic retroviruses, which were used to infect SCK cells. Transduced SCK cells were selected in media containing 400 ug/ml G418 (Gibco), and >200 colonies of surviving cells were pooled to maximize the likelihood of deriving lines that reflect the heterogeneity of the parental line. Flow cytometric analysis using a rat monoclonal antibody (mAb) to mB7 (Pharmagen, San Diego, CA) demonstrated that the MV6mB7-transduced SCK cells but not the MV6-transduced SCK cells expressed surface mB7.

mB7-SCK-MV6 cells and mB7+ SCK-MV6mB7 cells are indistinguishable morphologically and have similar in vitro growth characteristics, but differ significantly in their ability to form lethal tumors in syngeneic A/J mice. Injection of 5 x 10^3 viable mB7-was uniformally tumorigenic and lethal (6/6); whereas, 5 x 10^3 mB7+ SCK cells resulted in no mice developing lethal tumors (0/6). Since mice injected with 0.5 x 10^3 SCK cells consistently developed tumors, the survival of most mice receiving 5x10^3 mB7+ SCK cells is unlikely to be due to inadvertent injection of too few cells. These findings suggest that mB7 expression by SCK cells impairs their ability to form tumors in syngeneic hosts.

6. ADDITIONAL ANIMAL MODELS OF B7 MODIFICATION TO TREAT BREAST CANCER. Based on the recommendation of the original review of the grant, an animal model of breast cancer was developed to test the gene therapy proposed in the grant, before beginning the clinical trial in patients. Dr. Fred Chang's laboratory at the University of Michigan studied a mammary carcinoma, MT-7 in Balb/c mice. MT-7 is a cultured tumor cell line derived from a dimethylbenzanthracene-induced mammary carcinoma in the Balb/c host. A subline, MT-901, was derived from an early in vitro passage of cultured MT-7 tumor inoculated subcutaneously. MT-901 cells were determined to be weakly immunogenic in traditional immunization and challenge experiments. MT-901 cells that were genetically modified to express the co-stimulatory molecule B7-1 failed to generate tumors in two out of five mice that were inoculated subcutaneously, whereas five out of five mice had tumor growth when inoculated with the wild-type MT-901 tumor cells (54). MT-901 cells that were genetically modified to secrete GM-CSF also grew less well than wild-type MT-901 with no tumor growth in two out of five mice inoculated with a low GM-CSF secreting clone. In immunization and challenge experiments, neither genetic modification resulted in superior protection against a subsequent tumor challenge compared to wild-type tumor alone. In separate experiments, MT-901 cells that were genetically modified to secrete IL-12, initially grew, but then were rejected in all five mice that were inoculated. However, subsequent

challenge of these mice with wild-type tumor cells resulted in tumor growth in all the animals.

Zitvogel, Lotze and others at the University of Pittsburgh used a murine spontaneous mammary carcinoma, TS/A, that was modified to express B7-1 to inoculate mice and 80% remained tumor-free compared to 0-20% of mice inoculated with unmodified tumor cells (55). Two-thirds of the long-term tumor free mice were capable of rejecting a subsequent rechallenge with the wild-type tumor. In other experiments, the synergistic effect of mixing in IL-12 transduced tumor cells was clearly demonstrated.

Cayeux and others at Humboldt University also used the TS/A murine mammary carcinoma cell line and demonstrated that B7 transfectants failed to grow in 60-90% of the animals (56). Additional experiments to dissect the mechanism of action showed that indirect antigen presentation by host APCs (cross priming) as well as direct antigen presentation by the B7-1 modified tumor occurred.

Recently, Graham and colleagues at McMaster University used a direct intratumoral injection of a B7-1 adenoviral vector into tumors derived from a transgenic mouse mammary adenocarcinoma after 21 days of tumor growth when the tumors were visible (57). Although the injection of B7-1 virus alone did not result in shrinkage of tumor, it did cause pronounced growth delay and prolonged survival. In their model, only an adenoviral vector containing both IL-12 and B7-1 could cause regression of established tumor and cures.

In summary, additional studies by several investigators have extended the original observations about the value of gene modification of murine melanoma or sarcoma with B7-1 to murine models of breast carcinoma thereby strengthening the rationale for its use in human breast cancer patients. At the same time, while these additional experimental studies indicate that B7-1 gene modification may be necessary, by itself, it may not be the optimal way to achieve immune mediated tumor eradication.

BODY

This section of the annual report will summarize the work that has been performed on the grant during the entire four year grant period with specific reference to the tasks described in the Statement of Work.

TASK 1

Task 1 in the Statement of Work is to develop methods to isolate highly purified populations of breast cancer cells from tissue specimens of locally recurrent or metastatic sites obtained from patients with advanced, refractory breast cancer. The work is being conducted in the laboratory of Stephen P. Ethier, Ph.D. at the University of Michigan. The methods that were proposed in the original grant are described in detail below.

Specific Aim #1. Selective isolation of human breast cancer cells from primary tumors and metastases using monoclonal antibody-conjugated magnetic beads.
Rationale: In order to isolate a highly pure population of human breast cancer cells that are suitable for infection with adenoviral expression vectors it is necessary to prepare a viable single cell suspension of cells from breast cancer specimens. In our previous studies with primary and metastatic human breast cancer specimens we have used an enzymatic dissociation procedure to prepare breast cancer cells for cell culture experiments. For cell culture applications, generation of cell suspensions that consist of multi-cell aggregates of breast cancer cells and normal cells is sufficient and even advantageous in some ways. For the experiments to be performed in the present studies, the cell suspensions obtained following enzymatic dissociation of breast tissue specimens will be treated further to prepare single cell suspensions. Single cell suspensions prepared from these specimens will be exposed to a panel of monoclonal antibodies in order to separate normal cells from malignant cells. Finally, the isolated breast cancer cells will be infected with adenoviral expression vectors, containing either a reporter gene (LacZ) for developmental studies, or the B7 gene for gene therapy experiments.

a. Preparation of single cell suspensions of human breast cancer cells.
Solid tumor specimens, either primary tumors or solid metastatic nodules, will be minced with sterile scalpels until tissue pieces are approximately 1 mm^3. The minced tissues will be suspended in Medium 199 containing Worthington type III collagenase (Worthington Chemical Co., Freehold, NJ) at a concentration of 200 units per ml, and Dispase (Boehringer-Mannheim, Indianapolis, IN) at a concentration of 1 mg per ml. Twenty mls of enzyme solution are used per gram of tissue. The tissues are incubated overnight in a 37° water bath shaking at 65 rpm. The next day, the remaining tissue clumps are mechanically dissociated by repeated pipetting of the suspension. The cells are then washed three times by centrifugation at 250 x g and re-suspended in fresh Medium 199 after each wash. This enzymatic dissociation procedure results in a mixed suspension of single cells, small aggregates and large mammary organoids. The viability of the cells in this suspension is greater than 95%. To prepare a single cell suspension from the mixed aggregate population, the cells will be washed in Ca^{++}, Mg^{++}-free, Hanks balanced salt solution (CMF-Hanks BSS) and then incubated

for 4 hours in CMF-Hanks BSS containing 10 mM EDTA, at 4° with gentle rocking. The cells will be mechanically dissociated every hour during the four hour period by repeated pipetting of the cell suspension. If necessary to maintain viability of the cells during this incubation, the CMF-Hanks-EDTA solution will be supplemented with 5% fetal bovine serum that had been treated with Chelex to remove divalent cations. After the four hour incubation, single cells are separated from any remaining cellular aggregates by filtration through Nitex mesh with a 20 um pore size.

Our preliminary data indicate that collagenase/Dispase dissociation of breast tissues does not adversely effect the integrity of cell surface molecules as these aggregates are quite reactive to antibodies directed against cell surface proteins. In generation of single cell suspensions, however, it is imperative that a method be used that does not alter the peptide epitope present on the surface of breast cancer cells. For this reason, we have chosen to use chelating agents that disrupt cell to cell interactions without degrading cell surface molecules to achieve the final single cell suspensions. It is necessary to obtain single cell suspension for the final cell purification procedures as the separation methods make use of antibodies that bind to epitopes expressed on beast cancer cells and not on normal mammary epithelial cells. If cell aggregates are used in the cell isolation procedures and if aggregates contain both normal and neoplastic cells, then the purpose of using breast cancer specific antibodies would be defeated.

b. Isolation of breast cancer cells using antibody conjugated magnetic beads. The basic strategy for isolating breast cancer cells involves the use of magnetic beads (Dynabeads, Dynal Inc. Great Neck, NY) that have been conjugated with anti-mouse IgG antibodies. Thus, the anti-mouse antibodies on the beads can be bound to mouse monoclonal antibodies directed against cell surface epitopes to prepare a reagent that specifically binds cells expressing the epitope. Following incubation of a cell suspension with antibody coated magnetic beads, the bound cells can be separated from non-bound cells by placing the tube in magnetic particle concentrators (MPC) designed to hold microfuge tubes. The beads and bound cells adhere tightly to the wall of the MPC and the non-bound cells are aspirated from the tube. The tube is then removed from the MPC, the cells re-suspended in medium and this washing procedure is repeated three to four times. With this method, we have separated mixed cell populations with greater than 99% efficiency using antibodies against the erbB-2 protein and antibodies against breast epithelial mucins. The cells isolated in this way have been seeded into culture and exhibit high viability as indicated by their ability to attach in culture and proliferate.

To coat magnetic beads with mouse monoclonal antibodies, 1 x 10^8 anti-mouse IgG Dynabeads are suspended in 1 ml of CMF-Hanks BSS and incubated with 1 μg of mouse monoclonal antibody with rocking at room temperature for two hours. Following the incubation, the beads are washed extensively with CMF-Hanks BSS by adhering the beads to the tube wall using the MPC, aspirating the medium, re-suspending the beads in 1 ml of fresh medium and incubating with rocking for 30

minutes. This washing procedure is repeated three times. After the last wash, the beads are suspended in CMF-Hanks BSS at a concentration of 10^8 beads per ml.

To isolate cells using antibody conjugated magnetic beads, $2x10^7$ magnetic beads are added to a 1 ml aliquot of a cell suspension of $1x10^7$ cells and incubated with rocking at room temperature for two hours. Next, the cells bound to the beads are washed three times to separate them from non-bound cells. If necessary, the beads can be removed from the purified cells either by trypsinization or by incubation with the peptide epitope that was used to generate the primary antibody. The cells isolated in this way can be used to initiate cell cultures of breast cancer cells or can be infected with adenoviral expression vectors. For the experiments to be carried out in this project, we will employ magnetic beads coated with three different antibodies. The first antibody, Sm-3, was generated against the core peptide of breast epithelial mucins. As discussed earlier, altered glycosylation of mucins that occurs in greater than 90% of breast cancer cells reveals the peptide epitope that is masked in normal cells by glycosylation. Thus, the Sm-3 antibody coated beads will be the primary antibody for isolating human breast cancer cells from primary tumor specimens that contain both normal and neoplastic mammary epithelial cells. The Sm-3 antibody was obtained from Dr. Joy Burchell, Imperial Cancer Research Fund, London, UK. A second antibody, Mc-5, recognizes breast epithelial mucins expressed on virtually all breast cancer cells. This antibody also binds to normal mammary epithelial cells making it less useful for primary tumor specimens. However, magnetic beads coated with this antibody have been used in our laboratory to isolate breast cancer cells from metastatic lymph nodes and pleural effusion metastasis. This antibody was obtained from Dr. J. Peterson, Cancer Research Fund of Contra Costa, Walnut Creek, CA. Finally, a third antibody, Tab-254, binds to the extra-cellular domain of the erbB-2 protein. Magnetic beads conjugated with this antibody are useful in isolating breast cancer cells from primary or metastatic sites that overexpress the erbB-2 protein as a result of amplification of the c-erbB-2 (Her-2/neu) gene, which occurs in approximately 30% of breast cancer cases. A panel of Tab antibodies against the erbB-2 protein have been obtained from Dr. Beatrice Langton, Berlex Biosciences, Richmond, CA.

During the last four years, Dr. Stephen Ethier's laboratory developed improved methods for the isolation and cultivation of primary human breast cancer cells in vitro. His laboratory performed experiments to purify breast cancer cells that were only marginally successful. They used the Sm-3 antibody, which is reported to be luminal cell specific, attached to magnetic beads to purify cells. The overall methodology worked well, but the antibody was not specific enough and many cancer cells were not purified using this approach. They then conducted experiments with magnetic beads using antibodies to a cell surface marker that recognize an antigen, CALA, that is expressed by stromal cells and not expressed by luminal mammary epithelial cells or breast cancer cells. Magnetic beads were also coated with an antibody that recognizes mucins that are expressed by luminal cells and breast cancer cells. Thus, mixed cell populations obtained from primary tumors or pleural effusions were first incubated with CALA-coated beads to selectively remove stromal cells or mesothelial cells. The non-bound cells were then

either directly plated in culture or subjected to a second round of selection using beads coated with the mucin antibodies. These experiments demonstrated clearly that magnetic bead separation can be used with both primary tumor specimens and pleural effusion specimens to enrich for mammary epithelial cells of the luminal lineage. When used with pleural effusion specimens, this method results in nearly pure populations of breast cancer cells. When used with primary tumor specimens, this technique yields cultures enriched for normal luminal cells and breast cancer cells.

A second approach makes use of the observation that human breast cancer cells have a differential ability to survive under anchorage independent conditions compared to normal stromal or mammary epithelial cells. Using a microgravity cell generator to selectively isolate breast cancer cells, his lab has found that a 6 to 10 day culture in suspension results in death of the majority of normal cells and the survival of the breast cancer cells. It is expected that in future experiments, the antibody-magnetic bead approaches will be combined with the microgravity cell generator to obtain nearly pure populations of breast cancer cells.

His laboratory has now isolated and characterized ten new human breast cancer cell lines. Four of these cell lines are from primary tumors, one was isolated from a metastatic lymph node, one is from a skin metastasis, one was from a recurrent chest wall lesion and the remaining cell lines are from pleural effusion metastasis or malignant ascites. The cell lines express the range of oncogene changes known to occur in human breast cancer including; erbB-2 amplification and overexpression, overexpression of epidermal growth factor receptor, amplification of the FGFR 1 and 2 genes, mutations in P53 and alterations in pRB protein expression.

These lines represent the kind of range of human breast cancer cells that will need to be used in gene therapy studies. Furthermore, the isolation of these lines is indicative of the progress that his lab has made in developing methods and conditions for the routine isolation of human breast cancer cells. In addition to generating cell lines, his lab has shown that successful short term culture of human breast cancer cells is now possible for the majority of patient samples, and long-term cell culture of these breast cancer cells is possible with roughly 20% to 30% of specimens.

TASK 2

Task 2 in the Statement of Work is to study and optimize the efficiency of and longevity of B7 expression in breast cancer cells transduced with adenoviral vectors containing the human B7 cDNA. The work has been conducted in the laboratory of Stephen P. Ethier, Ph.D. at the University of Michigan. The methods that were proposed in the original grant are described in detail below.

> *Specific Aim #2. Study and optimize the efficiency and longevity of B7 expression in breast cancer cells transduced with adenoviral vectors containing the human B7 cDNA.*
>
> **Rationale:** Prior to the use of transduced breast cancer cells as immunotherapy the methodologies to transduce the maximum number of cells with an adenoviral vector

and to verify that large numbers of cells express the vector-encoded human gene for at least several days must be developed. In this specific aim, we will perform a series of experiments aimed at optimizing methods for the infection of purified human breast cancer cells with adenoviral expression vectors and for optimizing the expression of a transgene within these vectors. Experiments will also be performed to determine the immunogenicity of human breast cancer cells that express the B7 gene following infection with appropriate adenoviral expression vectors.

a. Infection of purified human breast cancer cells with adenoviral expression vectors. To perform the optimization experiments, an adenoviral vector containing a reporter gene (LacZ) as the transgene will be used. In preliminary studies with early-passage breast cancer cell lines developed in our laboratory, we have found that overnight exposure of these cells to these adenoviral vectors results in expression of the LacZ transgene in greater than 80% of the cells. To optimize infection of purified human breast cancer cells with the adeno-LacZ virus, aliquots of 10^6 human breast cancer cells, purified using methods described above, will be incubated in suspension with adeno-LacZ virus for 24 hours with gentle agitation. Multiplicity's of infection ranging from 10^2 to 10^4 pfu's per cell will be tested in these experiments. Following infection, cells will be seeded into culture using media that we have developed for human breast cancer cell growth, and the cells will be assessed for LacZ activity at 24 hours, 3, 7, 10 and 14 days after infection. This experiment will be carried out with cells from at least 10 separate breast cancer patients. In these experiments, we will determine the optimum multiplicity of infection and the duration of the transgene expression in purified human breast cancer cells infected with adenoviral vectors immediately after their isolation.

b. Infection of purified human breast cancer cells with B7-adenoviral vectors. Experiments will then be performed to transduce human breast cancer cells with the human B7 gene using adenoviral vectors developed by James Wilson and Steve Eck (see letter of consultancy). For these experiments the conditions shown to yield optimal transduction of the LacZ reported gene will be used for B7. Expression of B7 protein on the surface of the human breast cancer cells will be assessed by flow cytometry using CTLA4Ig or commercially available mouse anti-human B7 mAb. We will also verify that the cells are capable of supporting B7-mediated responses such as providing co-stimulatory signals (as assessed by proliferation and IL-2 gene expression) for autologous T lymphocytes activated with phorbol ester, bacterial superantigens, or PHA (methods as outlined under specific aim #4). Each of these is an accessory cell dependent stimulus, however we and others have shown that purified T cells can respond to these stimuli in the presence of B7-transfected CHO cells. Thus we will use B7-transfected CHO cells as a positive control in these studies. This also will allow us to compare the relative co-stimulatory abilities of $B7^+$ CHO cells and autologous $B7^+$ breast cancer cells. Since in the design of our phase I study (specific aim #3) the cells will be irradiated with 5000 cGy prior to injection into patients, we will also verify in these studies that the cells retain co-stimulatory capacity after irradiation at this dosage.

Dr. Ethier's lab performed experiments with an adenoviral expression vector containing the human B7-1 gene. This vector is essentially the same vector that will be used to prepare the autologous tumor cell vaccines for the clinical trial. The experiments performed thus far have made use of this vector (AdB7-1) and a series of human breast cancer lines that he has developed in his laboratory. There are a number of advantages of the cell lines he has developed. Since they were developed in his lab, they have all been tested in early passage, and thus are more representative of the breast cancer cell primary cultures that will eventually be used in the clinical trial. In addition, his lines come from a wide range of breast cancer specimens, ranging from early stage primary tumors, to large inflammatory primary tumors to metastatic specimens and chest wall recurrences.

To do these experiments, cells from the human breast cancer cell lines were cultured to high density and then infected for 2 hours with the AdB7.1 vector at various multiplicity's of infection (MOI). After 48 hours, the cells were harvested from the dish using 10 mM EDTA, and incubated with the high affinity B7 binding protein CTLA4Ig. The cells were then incubated with a fluorescent secondary antibody, washed extensively and then scanned by flow cytometry.

The FACs scans shown in figure 1 illustrate the detection of B7.1 expression in the human breast cancer cell lines 48 hours after infection. Table 1 shows the data obtained from these scans and indicates the percent B7 positive cells following infection at two different MOIs. These results indicate that all of the human breast cancer cell lines studied were successfully infected and expressed the B7 protein on the cell surface. Although there was some variability from experiment to experiment, these experiments indicated that at MOIs of 10^4 particles per cell, the vast majority of breast cancer cells express high levels of the protein on the cell surface. At lower MOIs, most of the breast cancer cells did express the B7 protein, but the proportion of positive cells was lower.

These results clearly indicate that human breast cancer cells from many different patients and derived from both primary and metastatic sites are successfully infected with the AdB7.1 vector and express high levels of the protein on the cell surface. In addition, the expression of B7.1 protein following infection is independent of the growth rate of the breast cancer cell lines. Indeed, SUM-44 cells, which still have doubling times of approximately 200 hours, are as readily infected as SUM-149 cells, which grow much more rapidly. Experiments currently underway are aimed at extending these experiments to studies with cells derived directly from patient samples, and examining the influence of radiation exposure on the long-term expression of B7 protein in these cells.

TASK 3

Task 3 in the Statement of Work is to conduct a Phase I clinical trial of B7 transduced breast cancer cells as a vaccine. Patients will be enrolled onto the clinical trial at the University of Michigan and at Portland Providence Medical Center. The detailed and complete phase I protocol was included in the original grant proposal. The methods that were proposed in the original grant are described in detail below.

Specific Aim #3. Conduct a Phase I clinical trial assessing the toxicity of autologous B7-transduced irradiated breast cancer cells as a vaccine to enhance the immune response to the tumor.

Rationale: In order to test the safety of a genetically modified autologous tumor vaccine, a phase I clinical trial must be performed. Although other cancer patients have received genetically altered tumor cell vaccinations (transfected with cytokine genes), this trial would represent one of the first times a genetically altered tumor cell vaccine that contained the gene for B7 was given. Therefore, it is prudent to begin the testing in patients with advanced refractory breast cancer. This is not the optimal setting for a vaccine to work, since these patients will have a significant tumor burden and have received several chemotherapy regimens, both of which can significantly depress the immune system. Ideally, a vaccine should be utilized to treat patients with low tumor burdens such as is the case after primary definitive surgery and/or irradiation. Nevertheless, the initial toxicity and safety studies must always be performed in patients with advanced disease and limited life expectancy. We plan to administer an autologous irradiated B7-transfected breast cancer cell vaccine once to patients with advanced breast cancer and observe them closely for side effects, toxicities, any clinical anti-tumor responses, and changes in their immune response to the tumor. Cohorts of six patients each will receive one of 3 dose levels (numbers of transfected breast cancer cells transfected) in an escalating fashion as toxicity permits. Assuming toxicity is not severe, future clinical trials could consider a vaccination schedule with several planned vaccinations, as well as the addition of adjuvants (if necessary) or systemic immune stimulating agents such as IL-2 following the vaccine. Future studies could also incorporate the harvest of regional lymph nodes and expansion of TILs for adoptive immunotherapy. The details of the proposed clinical trial are described in the model protocol that is in Appendix F. The key features will be delineated below. We recognize that certain technical details are still in the developmental phase. These details will be incorporated into the final version of the protocol prior to submission to the IRB, RAC and FDA. We also wish to emphasize that the appropriate animal toxicity studies will be performed prior to the initiation of the clinical trial by our collaborators Drs. Wilson and Eck (at the University of Pennsylvania) using the type of B7-expression vectors that would be used in our human studies. These toxicity experiments will include intravenous injection of the B7 vector, as well as deliberate transduction of a variety of non-malignant cells such as hepatocytes, fibroblasts and keratinocytes. The studies will assess the animals for adverse effects including the induction of unwanted autoimmune responses.

Phase I Study Objectives:

1. To determine the toxicity of subcutaneously administered irradiated autologous breast cancer cells that have been transfected with the human gene for B7 in patients with advanced or metastatic breast cancer.
2. To determine the maximum number of transfected breast cancer cells that can be safely given to these patients.
3. To determine if the vaccination results in an immune response and to characterize that immune response.
4. To observe patients for any antitumor responses.

Eligibility Requirements: Patients must have advanced breast cancer that has failed to respond to at least two standard chemotherapy regimens used in the metastatic setting and who are considered unlikely to benefit from further salvage chemotherapy regimens or hormonal regimens. They must also have a source of autologous tumor that can be easily harvested. This includes patients with subcutaneous or cutaneous metastases, patients with easily excisable lymph nodes containing metastatic tumor, and patients with malignant pleural effusions or ascites. Patients must have a good performance status and a life expectancy of at least three months. Patients must be at least 18 years old. *There is no exclusion for sex or ethnic background.* Patients must have evaluable or measurable disease in addition to the disease that will be surgically removed for the purposes of formulating the autologous vaccine. Adequate baseline organ function will be required. In addition, patients must not be anergic to standard recall antigens. Patients may not have received prior antitumor vaccines or immunotherapy. Patients will be excluded if they have any autoimmune diseases, evidence of HIV infection or AIDS, active infection, bleeding, pregnancy, or lactation, or any significant uncontrolled medical or pyschiatric illness. Patients who require corticosteroids or anticoagulation are ineligible.

Study Design: Patients will undergo surgical removal of metastatic disease under local anesthesia in order to provide autologous tumor cells that can be transfected with the human B7 gene. A section of the removed tumor will be sent to surgical pathology for pathologic diagnosis. The remainder of the specimen will go to the laboratory to prepare B7-transfected autologous tumor, for immunologic assays, and for cryopreservation. (For details on the purification and transfection of the breast cancer cells see the technical methods section for specific aim 1 and 2). After the autologous breast cancer cells have been transfected with B7 they will be irradiated with 5000 cGy, a dose of radiation that renders them nontumorigenic but allows them to remain metabolically active. They will then be injected intradermally into the thigh approximately 10 cm below the inguinal lymph nodes and the injection site will be marked with an ink tatoo for future biopsy. The injections will be administered in the Clinical Research Center, University of Michigan Hospital and the patients will remain in the hospital overnight. Cohorts of six patients each will be treated with escalating doses of autologous irradiated B7 transfected breast cancer cells according to the following scheme: a): 10^6 cells, b): 10^7 cells, c): 10^8 cells (for technical reasons, 10^8 cells is the likely maximum number of cells that could be obtained from these patients). Individual patients will receive one dose level of cells (i.e. there is no intrapatient dose escalation). Only one vaccination is planned for each patient unless the patient demonstrates clinical benefit from the treatment, whereupon the patient can receive additional monthly vaccinations as long as such benefit persists. Each patient will be observed for at least three weeks at a given level of cell injection before the patients are permitted to enroll on the next higher dose level of cells. If one or fewer patients experience dose-limiting toxicity at a given number of cells injected, escalation will be permitted to continue to the next level. If two or more patients sustain dose-limiting toxicity, then that level of cells will be determined as the dose-limiting number of cells and the dose level of cells below that will be

defined as the maximum tolerable dose of cells to be injected. It is possible that at the maximum dose of B7-transfected cells dose-limiting toxicity will not be observed.

Once escalation is completed, a separate cohort of six patients will be treated with both B7-transfected autologous irradiated breast cancer cells and vector only-transfected autologous irradiated tumor cells. One injection will be placed in the left thigh and one in the right thigh at the same time. The purpose of treating this cohort of patients is to compare the immunologic response at the vaccination site and in the draining lymph nodes from one leg to the other. This will help determine if B7 transfection enhances the immune response above that which is seen with transfection of vector alone.

Study endpoints:
TOXICITY: Patients will be closely followed and observed for the development of any clinical side effects from the treatment. Toxicity will be graded according to the Cancer Treatment Evaluation Program toxicity scale. The major toxicity that is anticipated is local redness, swelling, pain, and increased warmth at the injection site. Patients will be monitored for the development of clinical symptoms suggesting autoimmune disease or allergic reactions. Changes in laboratory parameters (complete blood count, chemistry panel, coagulation studies, urinalysis, as well as tests for the development of autoimmune disease [ANA, RF, CH50, anti-DNA abs, T4, TSH]) will be assessed two and four weeks after vaccination and thereafter once/month. In addition, sera and peripheral blood mononuclear cells will be obtained for archival purposes according to the current safety monitoring guidelines by the Center for Biologics Evaluation and Research (presently, once/month on treatment and every three months thereafter).

IMMUNE RESPONSE: A biopsy of the vaccination site along with surgical removal of one to three draining inguinal lymph nodes will be performed two weeks after the vaccination. Peripheral blood will be obtained at two weeks and 4 weeks and then once/month. DTH skin testing will be performed monthly. The details of the immunologic monitoring are extensively described in the methods section for specific aim #4.

ANTITUMOR RESPONSE: Four weeks after vaccination, the patients will undergo reevaluation to determine if their disease has responded or progressed using standard response criteria. Patients whose disease has not worsened or has regressed (even if it does not meet the criteria for partial regression) will be eligible to receive additional cycles of treatment using the autologous irradiated B7-transfected cancer cells providing that they experienced no severe toxicity with the first vaccination. Patients may continue this treatment until they have evidence of progressive disease.

Initiation of work on Task 3 is dependent on the development of a FDA-approved, replication-defective, recombinant adenovirus bearing a human B7 cDNA (Ad.hB7) for use in human clinical trials. Our subcontractors at the University of Pennsylvania, Dr. James Wilson, Dr. Stephen Eck, and Dr. William Lee informed us on October 30, 1998

(see attached lettter) that they had successfully produced a clinical grade vector for this clinical trial and that it had been made in compliance with Good Manufacturing Practice (GMP). The following is their summary of their experiments with this adenoviral vector:

Ad.hB7-1:
in vitro gene transfer: Ad.hB7-1 transduces a variety of mammary and melanoma cell lines in vitro to express hB7-1. Using antibody staining and flow cytometry, at an MOI of 10, about 32% of WM9 human melanoma cells express hB7-1 three days after transduction; at an MOI of 100, nearly 100% of WM9 cells express hB7-1 three days after transduction..

in vivo gene transfer: Ad.hB7-1 was injected into WM9 human melanoma tumors established in the flank of SCID mice. Flow cytometric analysis of single cell suspensions made from the injected tumors revealed that 26%, 19% and 32% of tumor cells expressed hB7-1 at 7, 14 and 21 days after injection, sequentially.

Cells transduced with Ad.hB7-1 deliver a co-stimulatory signal to human T cells: Ad.hB7-1-transduced or Ad.lacZ-transduced WM9 human melanoma cells (89%+ for hB7-1 and 94%+ for lacZ, respectively, at three days; WM9 cells constitutively express MHC class I and II antigens) were treated with mitomycin C and cocultured with purified, allogeneic human peripheral blood T cells. T cell proliferation stimulation indices calculated on the basis of ^{3}H-thymidine incorporation by the T cells indicated that there was a 33-fold greater stimulation of proliferation by Ad.hB7-1-transduced WM9 cells than by Ad.lacZ-transduced WM9 cells. There was also a 9-fold greater stimulation of proliferation by Ad.hB7-1-transduced WM9 cells than by untransduced WM9 cells. Similar results were obtained with Ad.hB7-1-transduced chinese hamster ovary (CHO) cells and WM793 human melanoma cells.

Ad.mB7-1:
in vitro gene transfer: Ad.mB7-1 transduces murine mammary and melanoma cell lines in vitro to express mB7-1 by flow cytometry. At an MOI of 1000, nearly 100% of K1735 murine melanoma cells express mB7-1 three days after transduction.

in vivo gene transfer: Ad.mB7-1 injected into subcutaneous K1735 tumors established in immunocompetent C3H/HeN mice resulted in tumor cell expression of mB7-1 determined by flow cytometry and immunohistochemical staining on days 3 and 7 but not on day 15. Extinction of expression is presumably due to immunological elimination of cells expressing adenovirus vector antigens, as has been described before.

Efficacy of mB7-1 delivered by Ad.mB7-1 in inducing tumor rejection: K1735 murine melanoma cells were transduced with Ad.mB7-1 or Ad.lacZ in vitro at an MOI of 1000. After three days, 10^{6} transduced or parental K1735 cells were injected into C3H/HeN mice. By 8 weeks, all 10 mice injected with parental K1735 cells developed progressive tumors, while 7/10 and 0/10 mice injected with Ad.lacZ- and Ad.mB7-1-transduced cells, respectively, developed progressive tumors. Mice

injected with parental K1735 cells developed tumors at a median of 22 days, while mice injected with Ad.lacZ-transduced cells developed tumors at a median of 36 days. When surviving mice were rechallenged with parental K1735 cells 8 weeks after initial challenge, 3/3 and 3/10 of the mice that had survived their challenge of Ad.lacZ- and Ad.mB7-1-transduced cells, respectively, developed progressive tumors (all 10 naive mice injected at the time of rechallenge developed progressive tumors). The results obtained with Ad.mB7-1-transduced K1735 cells are comparable to those achieved using retrovirus-transduced, B7-1+ K1735 cells (including the relatively low frequency of protective immunity at 8-12 weeks in mice that previously rejected B7-1+ K1735 cells). Efforts to assess the efficacy of in vivo Ad.mB7-1-transduced K1735 tumor cells (Ad.mB7-1 injection intratumorally) have been unsuccessful. In part, this may be due to the fact that treatment cannot begin until the subcutaneous tumors reach a size that can be injected - about 3-4 mm in diameter. At this point, the rate of unperturbed K1735 tumor growth is such that C3H/HeN mice live a median of 8-9 additional days before they die of tumor or require euthanasia which may be insufficient time for effective host immunization and tumor control.
Ad.mB7-1 efficiently transduces SCK mammary carcinoma cells in vitro to express mB7-1. Injection of 2.5×10^4 live Ad.lacZ- or Ad.mB7-1-transduced SCK cells into A/J mice resulted in 9/10 mice in both groups developing tumors, while 6/10 mice given retrovirus-transduced mB7-1+ SCK cells developed tumors. An adverse effect on outcome of adenovirus transduction itself was excluded by the fact that a similar fraction (6/10) of mice developed tumors that had been given retrovirus-transduced mB7-1+ SCK cells supertransduced with Ad.lacZ. A potential explanation for the lack of protection by Ad.mB7-1-transduced SCK cells may come from the constraints of working with this aggressive tumor model. We routinely inject 2.5×10^4 live SCK cells after which tumors usually appear in 6-8 days and the mice are dead in 13-15 days. When we introduce 2.5×10^4 live SCK cells made B7-1+ by stable retrovirus transduction, all progeny SCK cells express B7-1, the number of B7-1+ tumor cells expands and the effective immunization dose increases. In contrast, when we introduce 2.5×10^4 live SCK cells made B7-1+ by Ad.mB7-1 transduction, assuming one viral genome/transduced cell, only half of the progeny of B7-1+ SCK cells will express B7-1, the number of B7-1+ tumor cells never increases above 2.5×10^4 and the effective immunization dose remains constant and low. Injecting more live SCK cells (e.g. 10^5 or 10^6 cells) is not a solution because these larger inocula accelerate an already rapid disease course (death in 10-12 days) which we believe is inadequate to permit tumor immunization and immunological tumor rejection. Attempts to demonstrate the enhanced immunogenicity of Ad.mB7-1-transduced SCK cells by vaccination with 10^6 irradiated cells also have not succeeded, in part due to the enhanced immunogenicity of irradiated Ad.lacZ-transduced SCK tumor cells which may be preventing us from seeing an added benefit from vaccinating with mB7-1+, Ad-transduced cells.

The original Ad.hB7-1 vector being prepared for clinical development was found to have undergone an unexpected and unusual rearrangement. Rather than "correcting" the defect or trying to justify its clinical use, they decided to remake the vector. This set back the timetable by about 7 months (4 months to remake the vector and its

Ad.mB7-1 counterpart and go through 3 rounds of plaque purification; 3 months to prove the efficacy of the newly made Ad.hB7-1 and Ad.mB7-1 in vitro and in vivo).

A new Ad.hB7-1 vector was made with an Ad5 dl327 backbone and shown to be correct by restriction digests. It was shown to transduce cells to express immunoreactive human B7-1 (hB7-1) by monoclonal antibody and CTLA4-Ig staining and functional hB7-1 staining by costimulation assays using purified human peripheral blood T cells.

For preclinical toxicity and efficacy studies, a new Ad.mB7-1 vector was made in parallel with the Ad5 dl327 backbone and shown to be correct by restriction digests. The cDNA insert was sequenced and shown to encode normal mB7-1. It was shown to transduce cells to express immunoreactive murine B7-1 (mB7-1) by monoclonal antibody staining. Unfortunately, in vitro costimulation assays could not be used to test the function of the mB7-1 because splenic T cell preparations contained too many contaminating antigen presenting cells that expressed mB7-1 so that they proliferated too well without the addition of Ad transduced mB7-1+ cells. Thus, they used an in vivo assay of efficacy. K1735 murine melanoma cells transduced by Ad.mB7-1 or Ad.lacZ in vitro (> 90% cells transduced) were injected into naive, syngeneic C3H/HeN mice, and the latter monitored for tumor development. Whereas 10/10 mice injected with K1735 cells developed progressive tumors and 7/10 mice injected with K1735.Ad.lacZ cells developed tumors, 0/10 mice injected with K1735.Ad.mB7-1 cells developed tumors. Thus, tumor cell expression of mB7-1 conferred by Ad.mB7-1 infection induced their rejection. To determine the presence of protective antitumor immunity, the surviving mice were rechallenged with wild-type K1735 cells 60 days after their initial challenge. Whereas all naive mice given these cells developed tumors, 3/10 survivors of K1735.Ad.mB7-1 cells did not develop tumors. While a 30% incidence of protective immunity at 60 days seems low, this seems to be a characteristic of the K1735 model (58).

The mouse toxicity studies that the FDA requires prior to initiation of a clinical trial in humans were completed using the methods described as follows. In discussions with the FDA it was agreed that the toxicity study should be done with the mouse version of the adenoviral B7 vector rather than risk having an immune response against the human gene product that would result in some toxic response. The toxicity study examined the dose response to H5.030CMVmB7 in a total of 120 male and female C57BL/6 mice. The H5.030CMVmB7-1 vector was tested in a dose response study using three levels of the vector ranging from $5x10^9$ to $5x10^{11}$ particles/mouse delivered subcutaneously on the back. The animals were carefully monitored and any alteration in overall health was noted. At days 8, 22 and 60 blood was taken from a set of animals for each group and analyzed for liver function tests (ALT, AST, GGT, Alkaline Phos, and others) and for total blood cell counts (CBCs). On the necropsy day, the animals were weighed, sacrificed, gross lesions noted, and tissues prepared from 9 major organs and any gross lesions (for days 8 and 22) with a full tissue sampling for day 60 animals. Histopathological analyses were performed. Immunological tests on splenocytes on day 8 included: lymphoproliferative

responses, CD8+ cytotoxic T cell responses, and cytokine secretion patterns. Serum isolated from the remaining animals at day 29 was tested for neutralizing antibodies to adenovirus, and anti B7-1 antibodies. The results of these analyses were submitted to the FDA and the adenoviral B7 vector was approved for use in humans in the fourth quarter of 1998.

Now that appropriate personnel have been identified at the University of Michigan to conduct the clinical trial, the study will begin as soon as the vector is delivered. Human use approval will be obtained and the other necessary documentation submitted to the USAMRMC for appropriate action prior to the expansion of the clinical protocol to the subcontracting site in Portland, OR (The Earle A. Chiles Research Institute).

TASK 4

Task 4 in the statement of work is to perform immunological monitoring of the patients who receive the Ad.hB7-1 gene-modified autologous tumor vaccine. Work on this task will start once patient enrollment onto the clinical trial begins. The methods outlined in the original grant to accomplish this task are described below:

Specific Aim #4. Perform in vitro and in vivo immunologic monitoring studies on enrolled patients to assess the development of an anti-tumor immune response.

Rationale: It is our hope that the phase I clinical trial (specific aim #3) will demonstrate that administration of B7-transfected breast cancer cells is both safe and well tolerated. In addition to assessing safety, it is critically important to perform basic studies in order to determine if treatment resulted in the immunologic recognition of the tumor (even in the absence of a clinical response) and to dissect some of the mechanisms by which expression of B7 induces a host immune response in human breast cancer. Detecting an immune response would indicate that the theoretical basis for the strategy chosen was sound and this knowledge will lead us to continue our efforts to refine and improve the use of B7-transfected tumor cells.

Experimental Design: Immunologic parameters to be assessed include (1) in vivo testing for delayed-type hypersensitivity reactions (DTH) to irradiated tumor cells; (2) biopsies of vaccination sites and draining lymph nodes for histologic and immunohistochemical assessment as well as for RT-PCR detection of cytokine gene expression; (3) determination of peripheral blood T cell subsets; (4) examination of serum for the development of anti-tumor antibodies; (5) determination of in vitro responses of peripheral blood T cells and cells from regional lymph nodes to cryopreserved tumor cells as assessed by proliferation, cytokine production, and tumor cell lysis. The cryopreserved tumor cells to be used for these studies will be aliquots of the same B7-transfected and non-transfected cells which will be used for vaccination. However, the cryopreserved tumor cells will not be irradiated prior to freezing. Testing will be performed at the following time points:

	Pretx	2 wks	4 wks	8 wks	12 wks
DTH			X	X	X
Skin & LN Biopsies		X			
T cell subsets	X	X	X	X	X
Antibody screening	X	X	X	X	X
In vitro T cell assays	X	X	X	X	X

Experimental Methods:

A. Determination of T cell subsets.

Peripheral blood will be obtained by phlebotomy and mononuclear cells will be isolated by Ficoll-Hypaque density gradient centrifugation. Directly labeled monoclonal antibodies to human CD3, CD4, CD8, CD28, CD45RA, and CD45RO will be used for multi-color flow cytometry using various combinations of the commercially available antibodies conjugated to FITC, phycoerythrin, and PerCP. Cells will be stained using standard protocols and will be analyzed on a FACScan.

B. Antibody screening.

Serum samples from peripheral blood will be tested for antibodies directed against autologous B7-transduced and non-transduced breast cancer cells. Pre-treatment and post-treatment sera will be obtained as specified above, and stored at -80°C prior to assay, so that all samples from a given patient can be assayed simultaneously. Samples will be tested in serial dilutions for determination of antibody titer. The samples will be incubated with autologous B7-transduced or non-transduced breast cancer cells for 60 min on ice, washed three times, incubated with FITC-conjugated goat anti-human Ig, washed again, and analyzed by flow cytometry. Specificity of binding to breast cancer cells will be determined by screening serum against autologous T cells. To determine whether antibodies, if detected, are induced against only $B7^+$ cells, we will compare the results seen with $B7^+$ breast cancer cells and with non-transduced breast cancer cells. To determine if antibodies are directed at B7 itself, we will screen sera against $B7^+$ and $B7^-$ CHO cells. In positive serum samples, Ig isotype (IgM vs. IgG) will be determined by pre-treatment of samples with DTT to inactivate IgM antibodies.

C. Biopsies.

Biopsies of vaccination sites and of draining lymph nodes will be performed by Dr. Alfred Chang. Tissue samples will be processed for routine histologic staining to determine the presence of breast cancer cells and infiltrating lymphocytes or other inflammatory cells. Regional lymph nodes will be examined for evidence of immune responsiveness as assessed by hyperplasia. In addition, immunohistochemical staining will be performed for CD3, CD4, CD8, CD16 and CD28, to determine the phenotype of cells infiltrating the vaccination sites and draining lymph nodes and for HLA-class II and IL-2R to

determine the activational status of these cells. In the case of skin biopsies, we will also stain with anti-CD1a to determine whether Langerhans cells have been recruited into the injection site and for HLA-class I and II expression on tumor cells. Dr. Brian Nickoloff, who has extensive experience in the clinical and *in vitro* analysis of immunologic responses occurring in the skin will evaluate these tissue specimens. Specimens to be examined by immunofluorescent staining will be frozen in O.C.T. embedding compound, sectioned and stained for T cell subsets. Single color staining will be done by immunoperoxidase, as this will permit simultaneous evaluation of tissue histology. Two color staining will be accomplished with one antibody labeled with fluorescein, and the second labeled with biotin followed by streptavidin-Texas Red.

D. RT-PCR.

If the biopsy of the vaccination site contains infiltrating lymphocytes, we will use semi-quantitative reverse transcriptase-assisted PCR to determine whether cytokine mRNAs are present in biopsy sites. Cytokines to be studied will be IL-2, IL-4, IL-10, IL-12, GM-CSF, and IFNγ. RNA will be isolated using acid-phenol. Total RNA samples (1-5 μg) will be incubated for 10 min at 65° C, cooled for 3 min on ice, and reverse-transcribed into cDNA. The reverse transcription reactions will be then heat inactivated 95° C for 10 min and cooled for 3 min. The primers to be used for amplification of IL-2, IL-4, IL-10, and IFNγ, and the cycling conditions, have been previously described (84) and used by our laboratory for detection of cytokine mRNAs (Naidu et al., submitted). The IL-12 primers were synthesized based on published IL-12 cDNA sequence, and have been verified to be specific for IL-12 based on predicted size and specific hybridization with an internal oligonucleotide (Goodman et al., unpublished data). To ensure that differences in cytokine product amount correlate with differences in starting cDNA (and hence mRNA) amount, we will ensure that PCR amplification is in the exponential phase. This will be done by verifying that the intensity of the product bands (both cytokines and controls) is increasing for at least 3 consecutive PCR cycles and that their relative ratios are constant. After amplification, the PCR products will be resolved by electrophoresis in a 1.5% agarose gel blotted on a nylon membrane and hybridized to ^{32}P-labeled internal oligonucleotide probes (sequences indicated above). The membranes will then be analyzed on a phosphor-imager for accurate measurement of bound radioactivity. All reverse-transcribed RNA samples will be simultaneously amplified using β-actin and/or GAPDH primers to verify that the RNA was intact and that reverse transcription was successful. This will also permit the calculation of cytokine:control mRNA ratios for normalization between samples.

E. DTH.

Patients will be tested for DTH responses by intradermal injections of both irradiated autologous B7-transfected breast cancer cells and irradiated autologous nontransfected and vector-alone transfected breast cancer cells at

separate sites on the volar surfaces of both forearms. There will be three separate injections of 10^3, 10^4, and 10^5 cells on both forearms. Induration will be assessed 24 and 48 hours later. DTH responses to all three doses of autologous tumor cells will be measured and recorded as the largest biperpendicular diameters of induration at 24, 48, and 72 hour time points. A positive DTH test will be defined as induration measuring greater than 25 mm^2 overall (determined by multiplying biperpendicular diameters) as measured at any of the three time points. Control DTH responses will be tested simultaneously using Candida and/or mumps antigen. Although patients known to be anergic are excluded from the protocol, this will verify that non-responsiveness to breast cancer cells is not part of a generalized anergic state developing during, or as a consequence of, treatment.

F. In vitro T cell assays.

i. proliferation.

Mononuclear cells from peripheral blood or lymph nodes will be co-cultured with irradiated autologous B7-transfected and non-transfected tumor cells in 96-well tissue culture plates. Proliferation will be assessed by addition of ^{3}H-TdR (1 μCi/well) for the last 6 hours of a 6 day culture period. Negative control cultures will be wells containing mononuclear cells without tumor cells. Positive controls will be polyclonal mitogens such as anti-CD3 mAb, staphylococcal enterotoxin A and B, and PHA, and the duration of these cultures will be 72 hours. These will serve to verify that patients' lymphocytes are competent to respond to appropriate stimuli, and that lack of proliferation to tumor cells, if observed, is not part of a generalized immunosuppressive state. If a proliferative response to tumor cells is observed, we will determine whether or not CTLA4Ig and/or anti-B7 mAb (10 μg/ml) is capable of inhibiting this response. If so, this would indicate that this response is dependent on B7 expression in the relevant cells. Since these cultures will contain autologous antigen-presenting cells (known to express co-stimulatory signals such as B7), we will also isolate purified T cells from peripheral blood or lymph nodes and test their ability to proliferate to B7-transfected and non-transfected tumor cells. Our protocol using negative selection with monoclonal antibodies directed at B cells, macrophages, and NK cells (anti-CD14, anti-CD16, anti-CD19 plus anti-HLA DR) and magnetic beads is capable of producing highly purified (>99%) resting T cells devoid of functional accessory cells (85). The ability of purified T cells to respond to B7-transfected tumor cells would indicate that the tumor cell by itself is capable of inducing a T cell response, rather than providing a soluble neo-antigen which is presented by a $B7^+$ antigen-presenting cell. If purified T cells from patients vaccinated with B7-transfected tumor respond to non-transfected tumor cells, this would indicate that T cell activation in vivo by B7-expressing cells has led to the development of primed T cells which no longer require co-stimulation by B7 for their response.

ii. cytokine production.
Cultures conditions will be identical to those described above for proliferation studies. Culture supernatants will be frozen at -80°C for subsequent assay by ELISA for IL-2, IFNγ, IL-4, GM-CSF, and IL-10 using commercially available kits.

iii. tumor-cell lysis.
The ability of lymphocytes to lyse B7-transfected and non-transfected tumor cells will be ascertained by ^{51}Cr release. Briefly, tumor cells will be labeled with ^{51}Cr, and labeled target cells will be plated in 96-well round bottomed plates at 5000 cells/well. Varying number of washed effector cells ($0.25 - 0.5 \times 10^6$) will be added and will constitute the experimental well. The maximum or spontaneous release of ^{51}Cr will be determined by the addition of detergent, or of medium without effector cells, respectively. After 3.5 hours culture at 37°C, plates will be centrifuged and 0.1 ml of supernatant and counted in a gamma counter. Percent specific lysis is calculated as:

$$\frac{\text{(experimental cpm - spontaneous cpm)} \times 100}{\text{(maximum cpm - spontaneous cpm)}}$$

In these assays both B7-transfected and control-transfected tumor targets will be used as well as the NK target K562. When responses are observed, we will determine the ability of anti-CD4, anti-CD8, anti-HLA class I and anti-B7 mAbs to block the response. Initially, we will test T cells obtained immediately after harvest for cytotoxicity. If we fail to see any response, we will test cells which have been restimulated in vitro by 7-day co-culture with B7-transfected or control-transfected tumor cells.

ADDITIONAL EXPERIMENTS

The review of the original grant recommended that an animal model of breast cancer be developed to test the gene therapy proposed in the grant, before beginning the clinical trial in patients.

Dr. Fred Chang's laboratory at the University of Michigan studied a mammary carcinoma, MT-7 in Balb/c mice (54). MT-7 is a cultured tumor cell line derived from a dimethylbenzanthracene-induced mammary carcinoma in the Balb/c host. A subline, MT-901, was derived from an early in vitro passage of cultured MT-7 tumor inoculated subcutaneously. MT-901 cells were determined to be weakly immunogenic in traditional immunization and challenge experiments. MT-901 cells that were genetically modified to express the co-stimulatory molecule B7-1 failed to generate tumors in two out of five mice that were inoculated subcutaneously whereas five out of five mice had tumor growth when inoculated with the wild-type MT-901 tumor cells. MT-901 cells that were genetically modified to secrete GM-CSF also grew less well than wild-type MT-901 with no tumor growth in two out of five mice inoculated with a low GM-CSF secreting clone.

In immunization and challenge experiments neither genetic modification resulted in superior protection against a subsequent tumor challenge compared to wild-type tumor alone. In separate experiments, MT-901 cells that were genetically modified to secrete IL-12, initially grew, but then were rejected in all five mice that were inoculated. However, subsequent challenge of these mice with wild-type tumor cells resulted in tumor growth in all the animals.

The genetically modified MT-901 tumor cells were then tested for their ability to sensitize tumor draining lymph node cells (TDLN) for adoptive immunotherapy. The TDLN were harvested nine days after the inoculation of tumor cells and they were activated and expanded with anti-CD3 plus IL-2 in vitro and adoptively transferred into mice bearing three day established MT-901 pulmonary metastases. The B7-1 expressing clone induced pre-effector cells better than wild-type tumor in one of two experiments. The low GM-CSF secreting clone was no different than wild-type tumor, but the high GM-CSF secreting clone was significantly better than wild-type tumor in the induction of tumor reactive TDLN. In a similar but separate experiment, the IL-12 transfected clone failed to elicit pre-effector TDLN cells differently from wild-type tumor.

Subsequent experiments designed to determine the type of effector cell that mediated tumor regression with the successful GM-CSF secreting clone indicated that $CD4^+$ cells mediate the effect in this model. The activated CD4 cells expressed CD95L (*fas* ligand) and mediated the killing of MT901 tumor cells that were class II negative but CD95 positive. The CD4 mediated cytotoxicity was related to *fas* ligation because the addition of *fas* fusion protein inhibited the in vitro cytotoxicity of these $CD4^+$ cells (*J. Immunotherapy*, in press).

Dr. Chang's lab subsequently explored alternative approaches that could take advantage of B7-1 transgene expression, for example, by combining it with the co-expression of transgenes encoding for the cytokines GM-CSF or IL-12. His laboratory tested the effects of gene-modifying the poorly immunogenic D5 melanoma cell line with B7-1, IL-12, GM-CSF, as well as various combinations of the three. In this model, IL-12 and GM-CSF gene-modified tumor cells sensitized tumor draining lymph node cells that reduced the number of lung metastases when they were adoptively transferred to mice with three day old lung metastases. The use of tumor cells gene-modified with both IL-12 and GM-CSF was significantly better and was able to eradicate all the pulmonary metastases. The use of tumor cells gene-modified with both B7-1 and IL-12 or B7-1 and GM-CSF was not superior to IL-12 or GM-CSF alone in this model. The lack of effect of B7-1 in this model is consistent with a previous report demonstrating that the benefit of B7-1 gene-modification is observed in immunogenic tumors but not in poorly immunogenic tumors (59).

Because of the delay involved in obtaining the adenoviral vector from our subcontractor, Dr. Ethier's lab conducted experiments to test the suitability of other vectors for possible use in the clinical trial. Lipofection with a plasmid vector and delivery by a gene gun failed to get the LacZ gene into more than a small percentage of cultured human breast cancer cell lines and was therefore abandoned as a possible alternative strategy.

CONCLUSIONS

This final report summarizes the work performed during the last four years towards the goal of initiating a clinical trial in metastatic breast cancer patients of a tumor vaccine consisting of autologous tumor cells transduced with an adenoviral B7-1 vector. Success was demonstrated in developing techniques of purification of human breast cancer cells and in maintaining these fresh tumor cells viable in short term culture (Task 1). Excellent results were also obtained with the optimization of conditions to transduce human breast cancer cells with the adenoviral vector containing the human B7 gene (Task 2). Problems with the construction of the Ad.hB7-1 vector by our subcontractors necessitated remaking the vector, setting back the previous timetable and preventing the clinical trial from beginning during the grant award. The vector was successfully remade and experiments demonstrated that it transduces cells resulting in cell surface expression of human B7-1 capable of costimulating T cells. The Ad.mB7-1 vector was also remade and the toxicology studies of the Ad.mB7-1 vector in mice required by the FDA were completed. The FDA reviewed the compilation of all the pathologic and immunologic results, and approved the vector for clinical use in the fourth quarter of 1998. Although the clinical trial and the immunological monitoring of the patients on the trial (Task 3 and 4) were not performed during the timeframe of the grant award, these tasks will be accomplished now that the vector is available.

Although much of the planned research has yet to be conducted, the importance of the completed research is the demonstration that it is possible to isolate and culture (at least short term) breast cancer cells from fresh tissue surgically removed from patients. This capability permits a treatment strategy that utilizes patients' own tumor cells instead of a strategy that is dependent on only one antigen or on an allogeneic cell line. Using the patients' own tumor cells in an immunotherapy strategy permits the possibility of an immune response to multiple tumor antigens, some of which may be unique to the patient. Such a broad-based immune response to multiple antigens lessens the chance that the tumor could escape immune attack by mutation of a single antigen. The other implication of the completed research is that it is possible (at least in vitro and in animals) to use gene therapy to alter the cell surface molecules of breast cancer cells, thereby changing them from cells that the immune system ignores to cells that the immune system recognizes and can attack.

In addition, Dr. Chang's laboratory experiments demonstrated that gene modification of the tumor cell by GM-CSF produced superior results in his model of adoptively transferred tumor draining lymph node cells, indicating that alternative gene modifications of breast tumor cells might improve the immune recognition of the cancer and the therapeutic result of the immunotherapy. His work on the mechanism of tumor killing by *fas* ligation points to a very important way cancer cells could be attacked and killed by CD4+ effector cells even if the tumor is class II negative.

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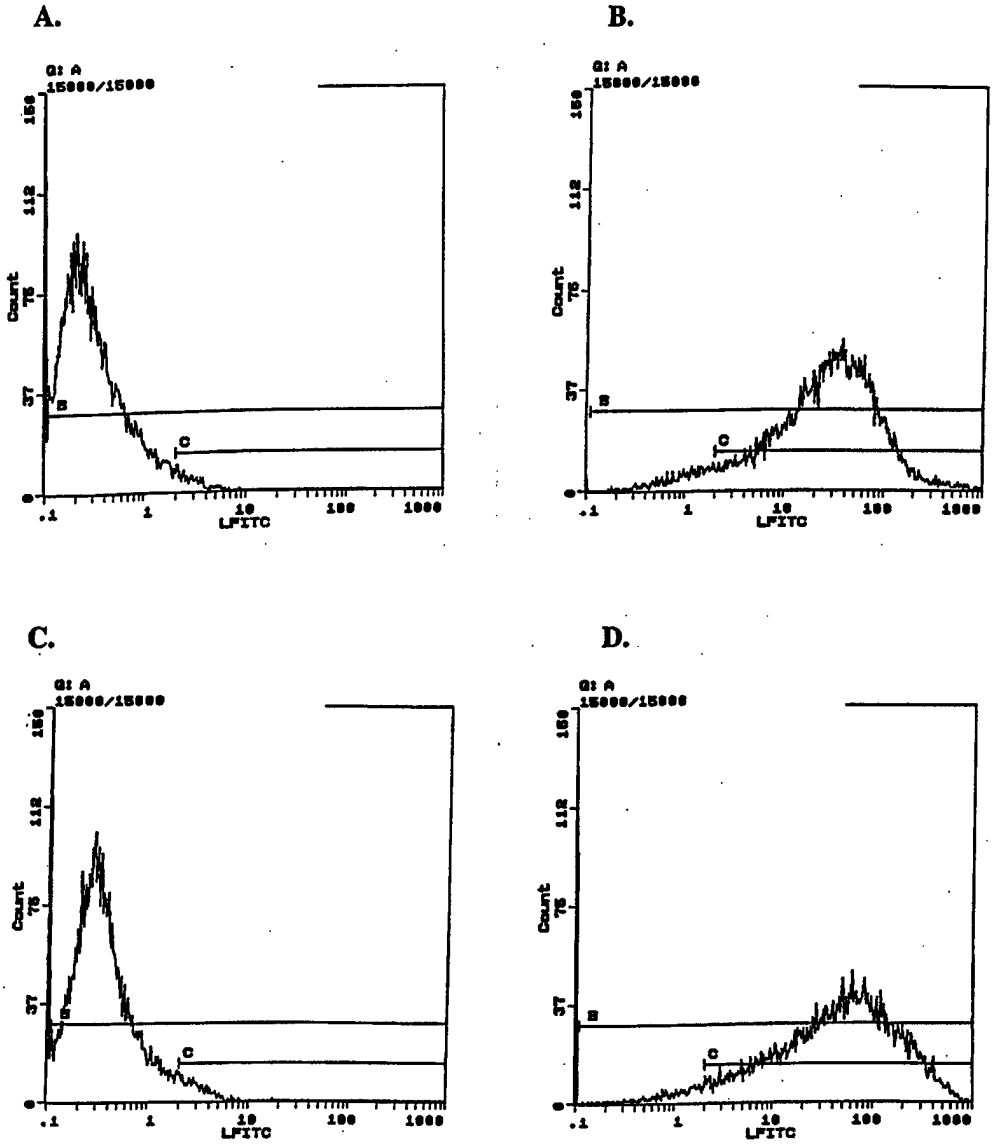
APPENDICES

Figure 1.



FACs analysis of B7.1 expression in two human breast cancer cell lines. Panels A and B show data from SUM-149 cells and panels C and D show data from SUM-159. Left hand panels show background fluorescence of control cells, and panels B and D show B7.1 specific fluorescence of AdB7.1 infected cells.

Table 1

B7 Expression in Human Breast Cancer Cell Lines

Cell Line	Multiplicity of infection (pfu/cell) 0	10^4	10^3
Sum-102	7.9	98.4	ND
	3.3	84.7	ND
Sum-149	4.3	92.8	ND
	5.1	75.6	38.9
Sum-159	3.0	92.9	ND
	12.4	97.9	ND
Sum-44	8.7	96.9	ND
	2.2	58.3	34.0
Sum-52	6.6	79.2	75.1

[a] percent B7-1 positive cells by flow cytometry

BIBLIOGRAPY OF ALL PUBLICATIONS AND MEETING ABSTRACTS RESULTING FROM THIS RESEARCH

1. Aruga, E., Aruga, A., Arca M.J., Lee, W.M., Yang, N-S., Smith II, J.W., Chang, A.E. Immune responsiveness to a murine mammary carcinoma modified to express B7-1, IL-12, or GM-CSF. Cancer Gene Therapy 4:157-166, 1997.
2. Tanigawa, K., Aruga, E., Aruga, A., Arai, H., Nickoloff, B.J.,Smith II, J.W., Chang, A.E. Characterization of the host immune response to mammary carcinoma cells modified to secrete GM-CSF. Abstract, presented at the DoD Breast Cancer Research Program: An Era of Hope, 1997.
3. Aruga, E., Tanigawa, K., Aruga, A., Arai, H., Smith II, J.W., Nickoloff, B.J., Nabel, G.J., Chang, A.E. CD95-mediated tumor recognition by CD4+ effector cells in a murine mammary model. J of Immunoth., 1999.

LIST OF PERSONNEL RECEIVING PAY FROM THIS EFFORT

John W. Smith II, M.D.
Alfred E. Chang, M.D.
Stephen P. Ethier, Ph.D.
Laurence A, Turka, M.D.
Etsuko Aruga, Ph.D.
Atsushi Aruga, Ph.D.
Keishi Tanigawa, Ph.D.
Hiroshi Arai, Ph.D.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Photocopy this page or follow this format for each person.

NAME	POSITION TITLE
Helen Alexandra Pass, M.D.	Assistant Professor of Surgery

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Michigan, Ann Arbor, MI	BS	1981-1984	Biomedical Sciences
University of Michigan, Ann Arbor, MI	MD	1983-1987	Medicine

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED TWO PAGES.**

Professional Experience

1987-1989	Intern/resident in General Surgery, University of Texas Health Science Center, Houston, TX
1989-1992	Medical Staff Fellow, Surgery Branch, NCI, National Institutes of Health, Bethesda, MD
1992-1994	Senior and Chief Surgical Resident, Georgetown Univeristy Hospital, Washington, DC
1994-1996	Senior Investigator, Surgery Branch, National Cancer Institute, NIH, Bethesda, MD
1996-present	Assistant Professor of Surgery, University of Michigan, Ann Arbor, MI
1998-present	Director, Breast Care Center, University of Michigan, Ann Arbor, MI

Publications

1. **Pogrebniak HW**, Stovroff M, Roth JA, Pass HI: Resection of pulmonary metastases from malignant melnoma; results of a 16 year study. *Ann Thor Surg* 46:20-23, 1988.
2. **Pogrebniak HW**, Mathhews WA, Chaudri G, Pass HI: Reactive oxygen species can amplify macrophage tumor necrosis factor production. *Surg Forum* XLI:101-103, 1990.
3. **Pogrebniak HW**, Roth JA, Steinberg S, Rosenberg SA, Pass HI: Re-operative pulmonary resection in patients with metastatic soft tissue sarcoma. *Ann Thor Surg* 52:197-203, 1990.
4. **Pogrebniak HW**, Matthew WA, Mitchell JB, Russo A, Samuni A, Pass HI: Spin trap protection from tumor necrosis factor toxicity. *J Surg Res* 52:197-203, 1990.
5. **Pogrebniak HW**, Matthews WA, Pass HI: chemotherapy amplifies production of tumor necrosis factor. *Surgery* 110:231-237, 1991.
6. **Pogrebniak HW**, Prewitt TW, Matthews WA, Pass HI: Tumor necrosis factor-α alters response of lung cancer cells to oxidative stress. *J Thor Cardiovasc Surg* 102:904-907, 1991.
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9. Chang K, Pai L, Pass HI, **Pogrebniak HW**, Tsao M, Pastan I, Willingham M: Monoclonal antibody K1 reacts with epithelial mesothelioma but not lung adenocarcinoma. *Am J Surg Pathol* 16:259-268, 1992.
10. **Pogrebniak HW**, Haas G, Linehan WM, Rosenberg SA, Pass HI: Renal cell carcinoma: Resection of solitary and multiple metastases. *Ann Thor Surg* 54:33-38, 1992.
11. Pass HI, **Pogrebniak HW**, Steinberg S, Mulshine J, Minna J: Randomized trial of neoadjuvant therapy for lung cancer: interim analysis. *Ann Thor Surg* 53:992-998, 1992.
12. **Pogrebniak HW**, Matthews WA, Pass HI: Decreased tumor necrosis factor production by Nicorandil. *J Surg Res* 52:395-400, 1992.
13. Jensen JC, **Pogrebniak HW**, Pass HI, et al: The role of tumor necrosis factor in oxygen toxicity. *J Appl Physiol* 72:1902-1907, 1992.

UNIVERSITY OF
PENNSYLVANIA
HEALTH SYSTEM

Institute for Human Gene Therapy

October 30, 1998

Dr. Alfred Chang
3302 Cancer/Geriatrics Center
1500 East Medical Center Drive
Ann Arbor, MI 48109

Dear Dr. Chang,

This is to inform you that the Institute for Human Gene Therapy (IHGT) at the University of Pennsylvania and their National Gene Vector Laboratory have produced vector for Michigan's use as per our subcontract to Michigan for DAMD17-94-J-4385.

The vector is adenovirus H5.030CMVhB7-1 and includes 3 production lots. The vector is clinical grade and made in compliance with Good Manufacturing Practice (GMP).

The direct costs of the vector is:

3 Production Lots	($18,150 each)	$54,450

The vector has been produced and is currently undergoing external Quality Control testing. As a result, we are submitting a copy of this as an invoice to Laurence Turka and his Business Administrator, Kathleen Bramwell (215) 227-3651 for payment of 50% now, with the balance of 50% due upon our shipment to you of the vector. As the grant, which covers this production, terminates at the end of this month, we will request that the 50% balance due be reported as an unliquidated obligation.

If you have any questions concerning the vector, its production, or testing, please contact Joseph Hughes (215) 614-0090 or Colleen Baker (215) 614-0094. You should expect to hear from them before the end of this calendar year that the vector has been tested and is ready for your use in clinical trials.

Thank you for your attention and consideration.

Sincerely,

Gale Thompson

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http://www.med.upenn.edu/~ihgt/